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Please provide the following references:

1) Structure Fold Des 2000 Jun 15;8(6):565-6
Re-engineering ketoacyl synthase specificity.
Val D, Banu G, Seshadri K, Lindqvist Y, Dehesh K.

2) J Biol Chem 1980 Dec 25;255(24):11949-56
Structural, enzymatic, and genetic studies of beta-ketoacyl-acyl carrier protein synthases I and II of Escherichia coli.
Garwin JL, Klages AL, Cronan JE Jr.

3) Chem Biol 1997 Oct;4(10):757-66
Molecular recognition of diketide substrates by a beta-ketoacyl-acyl carrier protein synthase domain within a bimodular polyketide synthase.
Chuck JA, McPherson M, Huang H, Jacobsen JR, Khosla C, Cane DE.

4) Curr Opin Biotechnol 1997 Aug;8(4):429-34
Engineering novel proteins by transfer of active sites to natural scaffolds.
Vita C.

Thank you,
David Steadman

Engineering novel proteins by transfer of active sites to natural scaffolds

Claudio Vita

Novel functional proteins have been generated by the transfer of active sites to structurally homologous proteins and to new structural contexts. The most successful examples of these approaches succeeded in providing effective new tools in biochemistry and protein chemistry and in suggesting new models in drug design.

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Abbreviations
CDR complementarity-determining region
IMDH isopropylmolate dehydrogenase

Introduction

Engineering novel proteins exhibiting a predetermined fold and a specific function is an exciting prospect of protein engineering. Nevertheless, owing to our limited understanding of the relations between structure and function, we are still a long way from being able to design new proteins with new functions at will. A traditional approach used to yield new functional proteins entailed modifying existing proteins by large scale random mutagenesis and (time consuming) screening of individual mutants of the protein of interest. An alternative, 'rational' approach to generating new binding or catalytic activities may involve the transfer of functional sites to appropriate natural structural scaffolds. Early successes, for example redesigning the specificity of a DNA-binding protein by exchanging the solvent-exposed functional residues of the 'recognition helix' with those of a homologous protein [1] and the swapping of the complementarity-determining region (CDR) loops from one antibody to another in order to transfer antigen recognition specificity [2], have definitively demonstrated that functional sites can be transferred from one protein to another, with conservation of structural integrity and gain in function. These examples had a seminal role in protein engineering and stimulated other applications, considered in this review. These 'transfers' will be described in three separate sections, corresponding to three conceptually different approaches: first, transfer of the functional residues from one protein to a structurally homologous one; second, transfer of a functional peptide sequence to a host protein structure (presentation scaffold), without considerations of structural homology but with the purpose of limiting the sequence flexibility; and third, transfer of a well-ordered active site to a different structural context to create a new function on an appropriate natural scaffold.

Transfer of active sites to homologous proteins

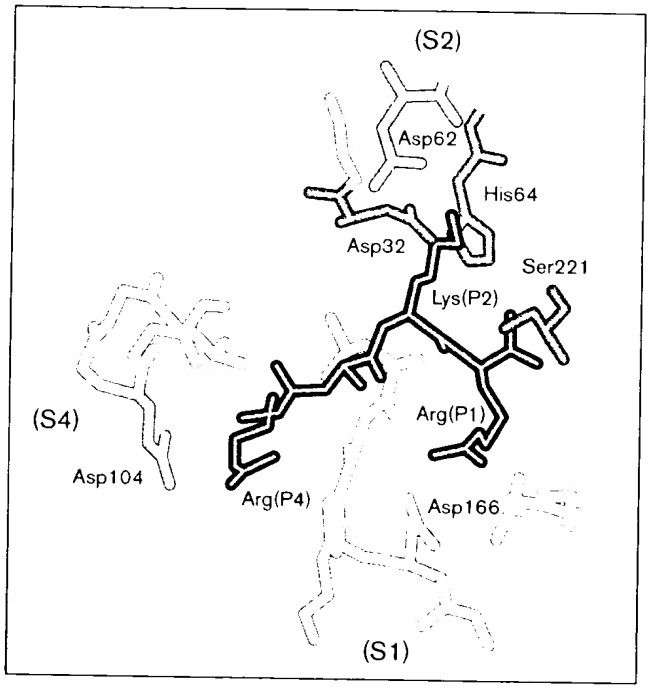
Changing substrate specificity

Serine proteases have been the subject of intensive engineering study aimed at changing their specificity. The examples reported illustrate how this was effectively realized by the transfer of appropriate functional elements from one protease to another homologous one. Trypsin and chymotrypsin have similar tertiary structures, with only four amino acid differences in the S1 substrate binding site (formed by residues 189–195, 215–220 and 225–228). Yet trypsin cleaves peptides at arginine and lysine residues and chymotrypsin prefers large hydrophobic residues. Replacement of the four divergent residues of the S1 site of cow trypsin with those of cow chymotrypsin is not sufficient to change the specificity for amide hydrolysis [3]. Trypsin is converted to a chymotrypsin-like protease, however, when two surface loops (residues 185–188 and 221–225) of chymotrypsin are also exchanged for the analogous trypsin loops [3]. These loops are not structural components of either the S1 binding site or the extended substrate binding sites: their effect is not on substrate binding but on the rate of the catalytic process, which they accelerate. Attempts to convert chymotrypsin to trypsin by the same means failed [4]: additional factors are probably involved in the substrate discrimination of trypsin and chymotrypsin.

Another example of recruiting substrate specificity from one member of a homologous gene family to another member by limited amino acid substitutions in the immediate vicinity of a bound substrate has been clearly demonstrated in the case of the subtilisin from *Bacillus amyloliquefaciens*. For example, the incorporation of *Bacillus licheliformis* substrate specificity into *B. amyloliquefaciens* subtilisin was obtained by exchanging only three residues, involved in van der Waals contacts with the substrate, with those of the *B. licheliformis* subtilisin [5]. More recently, subtilisin BPN′ was mutated to cleave substrates containing two consecutive basic (dibasic) residues [6] and three basic (tribasic) residues [7••]. Mutants were designed on the basis of the structure of subtilisin BPN′ and by considering sequence differences between it and the eukaryotic homologs Kex2 and furin, which are known to cleave dibasic and tribasic substrates, respectively. The incorporation of just two acidic residues, found in the eukaryotic enzymes and proposed to interact with the dibasic substrate, at the positions P_1 and P_2 efficiently

shifted the specificity towards basic residues. An additional specificity for basic residues at the P_4 position of the substrate was then engineered [7••], by introducing a subtilisin to furin single substitution at the S4 subsite. The novel protease, called furilisin, cleaves tribasic substrates (based on the RAKR or KAKR amino acid sequence [single-letter code]) with high catalytic efficiency and specificity (Figure 1). These studies provide a basic example of how to manipulate substrate specificity in a modular fashion, thereby creating a new enzyme that may be a useful tool to cleave at engineered tribasic linker sequences between proteins and fused affinity tags.

Figure 1



Model of an Arg–Ala–Lys–Arg peptide bound to furilisin. The novel protease contains only three acidic substitutions, Asp166, Asp62 and Asp104 from furin S1, S2 and S4 sites, that are supposed to interact with the basic residues of the P1(Arg), P2(Lys) and P4 (Arg) sites, respectively, of the substrate. Interestingly, the cumulative effect of incorporating acidic residues in three separate enzyme subsites had a substantial synergistic effect on the specifity for basic residues. (Adapted from [7••].)

Changing coenzyme specificity

The first successful examples of a change in coenzyme specificity was reported for the glutathione reductase [8,9] and in the lipoamide dehydrogenase [10] from *Escherichia coli*, two dehydrogenases containing the typical Rossman fold. The natural preference of the first for NADP and of the second for NAD was inverted by limited amino acid substitutions (exchange of critical functional residues) in the coenzyme-binding domain on the basis of the known structure of the two enzymes. Inverting the NAD preference of the *Thermus thermophilus* isopropylmalate dehydrogenase (IMDH), however, presented a singular challenge [11••]. Comparison with the NADP-dependent *E. coli* isocitrate dehydrogenase revealed that a β turn in the NAD-binding pocket of IMDH is replaced by an α helix in IDH; thus, success critically depended on being able to engineer secondary structures in the enzyme binding site. Accordingly, the 13-residue α helix of the *E. coli* isocitrate dehydrogenase was modeled in place of the seven residues constituting the β turn in the *T. thermophilus* IMDH: four substitutions were suggested by this model to avoid steric packing problems, together with four additional substitutions to stabilize the binding pocket. The engineered dehydrogenase showed a shift of preference from NAD to NADP by a factor of 100,000 fold [11••]. This example demonstrates that the active site transfer strategy had to be combined with structure modeling to provide novel properties in some enzymes.

Changing binding activity

Engineering a new binding activity by the transfer of functional loops appears to be more straightforward than an intervention in the active site of an enzyme. The Cys_2His_2 zinc finger represents a particularly attractive motif for protein engineering, since it is well characterized structurally and has distinct DNA binding properties specified by the solvent exposed region on the helix part of the molecule. Controlled DNA-binding properties have been introduced in the zinc-finger framework by substituting seven residues of the solvent exposed face of the helix with those taken from different zinc fingers [12]. This and other work reporting the display of the zinc finger module on phages has permitted the derivatization of specificity rules for the design of new DNA binding proteins (reviewed in [13]).

By transferring eight functional residues of human growth hormone to human prolactin, Wells and co-workers [14] engineered a prolactin able to bind to the growth hormone receptor. Such hybrid hormones could be useful as receptor agonists to separate receptor binding and activation processes. The eight residues to be transferred, however, were not determined solely on a structural basis, but the choice was made after seven rounds of site-directed mutagenesis and functional assays. This work is important because it demonstrates the feasibility of recruiting receptor-binding properties from distantly related and functionally divergent hormones, and also emphasizes that a detailed functional analysis is crucial to guide the design of a protein–protein interface.

The structural homology between angiogenin and ribonuclease A has been at the basis of interesting loop exchange experiments [15,16], demonstrating that unrelated activities can be endowed in a suitable structural platform simply by the transfer of a single loop. A 13 residue surface loop of angiogenin has been replaced with the corresponding 15-residue loop of pancreatic

ribonuclease A [15]; the *in vivo* angiogenic potency of the hybrid is markedly decreased, while the enzymatic (ribonuclease) activity is dramatically increased. In a complementary experiment, the same 15-residue surface loop of ribonuclease A has been substituted by the corresponding 13-residue loop of angiogenin [16]: the hybrid showed angiogenic activity comparable to authentic angiogenin and a reduced ribonuclease activity.

Another, more recent, successful example of loop transfer illustrates the potential for changing the receptor specificity of a protein ligand. Basic fibroblast growth factor, presenting a β-barrel structure similar to that of interleukin-1β, was made to bind with high affinity to the receptor for acidic fibroblast growth factor, simply by replacing a five-residue surface loop with the corresponding seven-residue loop from the structural homologue acidic fibroblast growth factor [17].

Transfer of functional sequences to presentation scaffolds

Functional peptide sequences have been transferred, mainly as insertions, to permissive regions of a globular protein as a way to limit conformational flexibility and to present the sequence in a controlled structural context. Antigenic epitopes, for example, were inserted within different recipient bacterial proteins as a means to induce antibodies against a chosen peptide sequence, without the need to synthesize the peptide. These genetic constructions, more homogeneous than the hybrids obtained by chemical coupling and easily purified by affinity chromatography using a specific affinity of the carrier protein, provide stimulation of T cells through T-cell-specific determinants [18–20]. Antigenic sequences were also inserted in the antibody CDRs, as a means of restricting their conformation and of providing an immunogenic response [21,22]; this process, called 'antigenization' of antibodies, is conceptually different and opposite from that called 'humanization' of antibodies [2], which implies the exchange of CDR loops from one antibody to another in order to transfer antigen recognition specificity and to reduce the immune response.

The amino acid sequence RGD (single-letter code) is present in a number of cell adhesion proteins (fibronectin, vitronectin, von Willebrand factor and fibrinogen) and is used for recognition by cell surface receptors (integrins). The concept of installing this simple tripeptide sequence in different proteins of known three-dimensional structure has been used by many groups as a way of fixing a conformation that may determine increased or specific binding to integrin receptors. The RGD motif, included in a sequence of varying length, was inserted into a long solvent-exposed loop of lysozyme; the construct possessed a new cell adhesion activity [23]. In a subsequent design [24], the adhesive sequence was flanked by two cysteine residues to form a disulfide bridge and to restrict further the conformation of the inserted sequence; the new lysozyme hybrid showed a cell adhesion activity, which was approximately 5–10% of vitronectin activity [24]. Analysis of the three-dimensional structure of this construct revealed that the RGD region was well defined and assumed a type II′ β turn conformation, with the arginine and aspartic acid sidechains pointing in opposite directions; this conformation was suggested to be essential for binding to integrin with high affinity [25]. Two other groups [26,27] used the framework of IgG to present the RGD sequence for integrin binding and used the CDR3 loops for the insertion, obtaining molecules showing high affinity for the fibrinogen receptor. X-ray three-dimensional structure analysis of one of these constructions [28] revealed that the RGD region was well defined with a 'turn-extended-turn' conformation, and with the arginine and aspartate acid sidechains pointing in opposite directions, a conformation not too dissimilar from that shown by the conformationally constrained loop inserted in human lysozyme [25]. The authors of [28] found that the RGD conformation had features in common with that of constrained peptide and nonpeptide RGD mimetics, known to bind to the fibrinogen receptor. This work confirms the utility of structural information derived from sequences installed on presentation scaffolds in the search for effective peptidomimetic templates, and validates the approach of presentation scaffolds in drug discovery.

An interesting application of a small and well-structured protein, the chymotrypsin inhibitor 2 from barley seeds, as a presentation scaffold of a Gln_{10} repeat has been recently reported [29]. The sequence was inserted into a long and flexible solvent-exposed loop of the scaffold; this insertion caused the molecule to form dimers and trimers by association of the glutamine repeats in β-pleated sheets. These results are in agreement with the hypothesis that such repeats, by linking long glutamine stretches by hydrogen bonds and provoking protein aggregation, are the cause of inherited neurodegenerative diseases, like the Huntington and Kennedy diseases [29].

Transfer of active sites to new structural contexts to create novel proteins

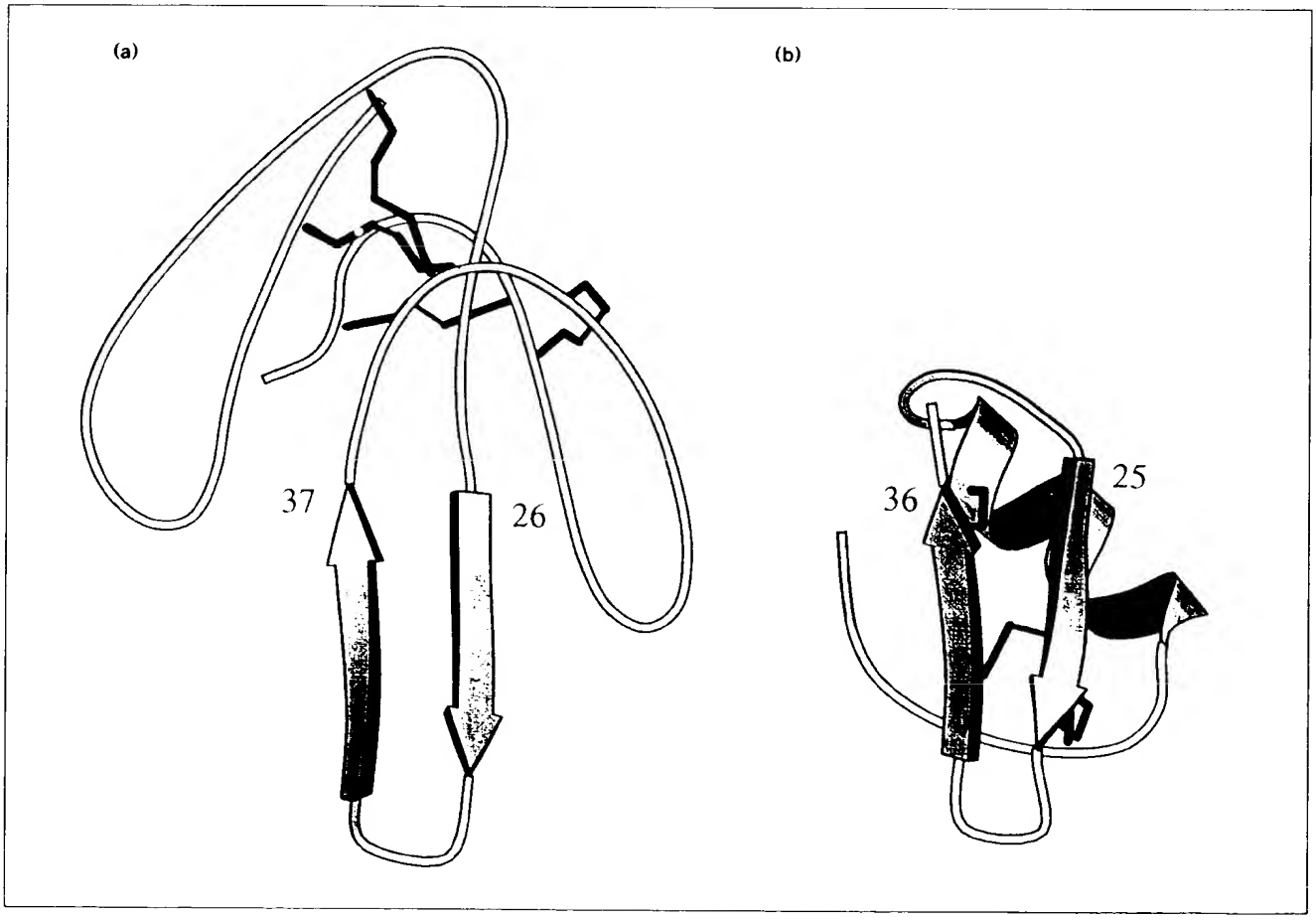
Few examples have been reported of the transfer of well-ordered active sites to structurally unrelated proteins with conservation of the structure and function of the transferred sites. Hynes *et al.* [30] were the first to demonstrate that a β turn of the staphylococcal nuclease (residues 27–31) can be substituted by a turn sequence (residues 160–165) from concanavalin A and that, in the resulting hybrid protein, the guest turn sequence retained the conformation present in the parent concanavalin A structure. No structural homology is present between staphylococcal nuclease and concanavalin A and the transfer operation was performed uniquely on the basis of a good alignment between the β strands leading away from the turn in the guest and in the host protein. This experiment clearly suggests that β turns

may be transferred from one protein context to another as structural 'cassettes'. In a similar approach, the inhibitory loop from the soybean trypsin inhibitor (Kunitz) and from other protease inhibitors was transferred into the interleukin-1β structure in place of a tight turn: this transfer operation conferred on the chimeric cytokine specific protease sensitivity or inhibition [31].

In a more recent example, a β hairpin, representing part of the site by which curare-mimetic snake neurotoxins bind nicotinic acetylcholine receptors, has been transferred to the α/β fold of the scorpion charybdotoxin (Figure 2) [32••], a scaffold particularly stable and permissive for sequence mutations [33]. The resulting chimeric protein binds to the acetylcholine receptor, although with a relatively low affinity. Structure resolution of this chimera by ^{1}H-NMR [34] revealed that the transferred site is stabilized by the structural scaffold in a conformation similar to that present in the parent neurotoxin, suggesting that the strategy of active site transfer to a stable scaffold has general applications in the engineering of novel ligands for membrane receptors.

In another example, the transfer of a biologically active loop to a new structural context resulted in an active ligand for the platelet integrin $\alpha_{IIb}\beta_3$ [35••]. The CDR3 loop of a monoclonal antibody selected to bind the integrin receptor $\alpha_{IIb}\beta_3$ with nanomolar affinity was grafted onto the epidermal growth factor-like module of human tissue-type plasminogen activator. Specifically, the guest loop was grafted within a disulfide-stabilized β turn exposed on the protein surface. The resulting chimeric protein bound the platelet receptor with nanomolar affinity and retained full enzymatic activity. Since the transferred loop sequence was derived from that of an antibody subjected to 'affinity maturation' using a phage display system [36], these results suggest that phage display can be combined with loop transfer to direct proteins to selected biological targets. This approach will eliminate the need to create new libraries for each protein studied.

Figure 2



Three-dimensional structure of **(a)** the curaremimetic neurotoxin toxin α and **(b)** the scorpion scaffold charybdotoxin. The representation emphasizes the central loop of the neurotoxin that has been transferred to the scorpion scaffold to engineer the curaremimetic chimera. The figure was generated from the 1nea (toxin α) and 2crd (charybdotoxin) coordinates in the Brookhaven protein data bank, using the software Molscript. (Adapted from [32••].)

Conclusions

The studies described in this review have demonstrated that transfer of active sites to natural scaffolds is effective in producing new functional proteins, representing interesting models useful in the understanding of structural determinants of specificity in substrate–enzyme, coenzyme–enzyme, DNA–protein, ligand–receptor and protein–protein interactions. In some cases, the new proteins, because of their new specificities, have been shown to be valuable tools in biochemistry and protein chemistry, and to represent new templates useful in drug design and discovery.

Furthermore, the concept of using a new structural context to induce a well-defined conformation of a specified sequence or to stabilize the structure of a predetermined active site seems to be quite promising. The results obtained in these transfer operations clearly suggest that, in the future, it should be possible to construct a family of well-defined structural domains presenting a wide set of structural secondary and tertiary motifs, onto which active sequences can be transferred and new functions generated. Stably expressed on the surface of phage, the transferred sequences can be rapidly changed, on the basis of screening assays, to increase their biological potency to yield conformationally well-defined artificial proteins active against specific biological targets.

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- •• of outstanding interest

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